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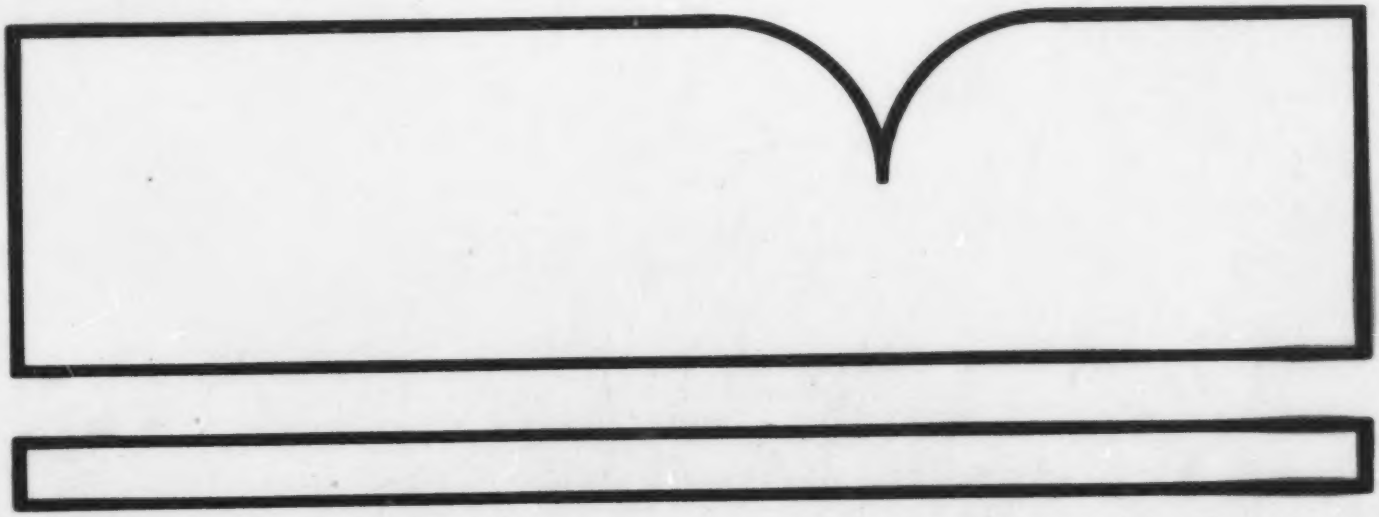

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METHODS FOR THE RECOVERY OF ORGANIC CARCINOGENS FROM WATER

by

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16. ABSTRACT

The organic carcinogens benzo(a)pyrene, dieldrin, and N-acetyl-2-aminofluorene were recovered on XAD-2 macroreticular resin in yields of 90 percent or more from distilled water or seawater and in yields of 40 percent or more from Lake Pontchartrain water containing a high concentration of organic material. The original solutions contained less than 500 parts per trillion of carcinogen. These results show that XAD-2 provides an efficient means for recovering nonpolar organic carcinogens from dilute solutions. More polar carcinogens such as dimethylnitrosamine were not effectively recovered on XAD-2 columns.

Since XAD-2 binding would not be selective for carcinogens, we investigated methods which might bind carcinogens selectively from a mixture of organic compounds. We tested the ability of the above carcinogens to bind to nucleic acid using direct binding, equilibrium dialysis, nuclei binding, and binding to DNA-cellulose. Radiolabeled carcinogens were used to quantify the amount bound. Either rat liver nuclei (0.1 mg DNA) or DNA-cellulose (1 mg DNA) bound 18 percent of the acetylaminofluorene and up to 66 percent of the dieldrin from solutions containing 150 to 280 nmoles of compound. Up to 30 percent of the benzo(a)pyrene from solutions containing as much as 320 pmoles was bound. Ten-fold or lower recoveries were found when direct-binding or equilibrium-binding methods were used.

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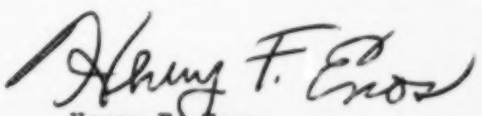
FOREWORD

The protection of our estuarine and coastal areas from damage caused by toxic organic pollutants requires that regulations restricting the introduction of these compounds into the environment be formulated on a sound scientific basis. Accurate information describing dose-response relationships for organisms and ecosystems under varying condititons is required. The Environmental Research Laboratory, Gulf Breeze, contributes to this information through research programs aimed at determining:

- the effects of toxic organic pollutants on individual species and communities of organisms;
- the effects of toxic organics on ecosystems processes and components;
- the significance of chemical carcinogens in the estuarine and marine environments;

The concentration and separation of polluting substances from estuarine water for purpose of indentification and hazard evaluations represent a significant technical problem. This report describes research to develop simple methods useful in separating trace amounts of organic carcinogens from marine waters. A unique approach to the separation of non-polar organic carcinogens from other non-polar organic compounds was explored. Methods such as these will be useful to the Agency in assessing the potentical hazard of organic compounds introduced into the estuarine environments.



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ABSTRACT

The organic carcinogens benzo(a)pyrene, dieldrin, and N-acetyl-2-aminofluorene were recovered on XAD-2 macroreticular resin in yields of 90 percent or more from distilled water or seawater and in yields of 40 percent or more from Lake Pontchartrain water containing a high concentration of organic material. The original solutions contained less than 500 parts per trillion of carcinogen. These results show that XAD-2 provides an efficient means for recovering nonpolar organic carcinogens from dilute solutions. More polar carcinogens such as dimethylnitrosamine were not effectively recovered on XAD-2 columns.

Since XAD-2 binding would not be selective for carcinogens, we investigated methods which might bind carcinogens selectively from a mixture of organic compounds. We tested the ability of the above carcinogens to bind to nucleic acid both before and after S9 liver microsomal activation. The DNA-carcinogen interaction systems included direct binding, equilibrium dialysis, nuclei binding, and binding to DNA-cellulose. Radiolabeled carcinogens were used to quantify the amount bound. Either rat liver nuclei (0.1 mg DNA) or DNA-cellulose (1 mg DNA) bound is percent of the acetylaminofluorene and up to 66 percent of the dieldrin from solutions containing 150 to 280 nmoles of compound. Up to 30 percent of the benzo(a)pyrene from solutions containing as much as 320 pmoles was bound. Ten-fold or lower recoveries were found when direct-binding or equilibrium-binding methods were used. When liver microsomes were used for activation in the binding systems, less carcinogen was recovered with the DNA fraction. In these cases, the microsomal protein can decrease the net DNA binding by competing with the DNA for carcinogen or by converting the carcinogen to products which do not bind well to DNA.

In the nuclei bindings studies, DNA was isolated from the nuclei by extraction with sodium dodecyl sulfate and phenol followed by recovery of the DNA by ethanol precipitation and spooling. The recovered DNA contained more than 10 percent of the input radioactivity, indicating that a significant portion of the input sample may be tightly bound to DNA. The portion which bound after DNA isolation was not dissociated by further extractions with chloroform-isoamyl alcohol or 1 percent sodium dodecyl sulfate solution. At least some of this material may be covalently associated. Whether or not DNA-binding is more specific for recovery of organic carcinogens than is XAD-2 remains to be explored.

This report is submitted in fulfillment of Contract No. R805656 by Gulf South Research Institute under the sponsorship of the U.S. Environmental Protection Agency. This report covers the period November 1, 1977, to October 31, 1978, and work was completed December 15, 1978.

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SECTION 1

INTRODUCTION

Estuarine waters are subject to pollution from oil spills and influx of industrial effluents from rivers. The pollutants can be directly toxic to marine life or can be concentrated by marine organisms which are then eaten by fish or man and thereby indirectly or directly rendered dangerous to human health. The concentration and separation of substances from estuarine water for the purpose of establishing their existence and hazard represent a significant problem since the substances are diluted by large quantities of saline water.

The generalized parameters typically used to characterize water (biological oxygen demand, total organic carbon, etc.) represent only a cross-section of the materials that raw and treated industrial waste effluents may present to estuarine water bodies. The application of these generalized criteria for assessment of the danger to man and the environment cannot necessarily protect a given section of the population or environment from hazardous or toxic compounds.

Novel methods are needed for further isolation and analysis of the most dangerous substances, since dilution of these substances in the water

prevents adequate characterization. In addition, due to the complexity of the mixture, one method of characterization could interfere with another. For example, characterization of a mutagen by the Ames Salmonella/microsome test could be prevented by the presence, in the mixture, of chemicals toxic to the test bacteria. Also, certain substances could poison the microsomal system and prevent activation of a premutagen to a mutagen. Techniques such as gas chromatography/mass spectrometry, while extremely sensitive, are very costly and do not provide an assessment of the danger of a particular mixture to animals.

Our goal in the present research has been to investigate simple methods which might be useful in separating organic carcinogens (polycyclic aromatic hydrocarbons, in particular) from dilute solutions and in further separating these from nontoxic, or toxic but noncarcinogenic, substances. For the initial separation of nonpolar organic substances from water, XAD-2 binding was used due to its low cost and ability to achieve high recoveries. The presumed ability of carcinogens to bind to DNA has been investigated as a means of separating such species from other nonpolar organics. In the present studies, only radioactively labeled, pure carcinogens have been tested for XAD-2 and DNA binding. Additional studies are needed in which actual samples obtained from estuarine waters are tested.

SECTION 2

CONCLUSIONS

The polycyclic hydrocarbons dieldrin, benzo(a)pyrene, and N-acetyl-2-aminofluorene bind well to XAD-2 resin and can be recovered from very dilute solutions in impure waters in yields greater than 50 percent. Dimethylnitrosamine was poorly recovered under these conditions. All of these compounds were strongly adsorbed to nuclei or DNA-cellulose and less strongly to DNA in direct binding studies. From 10 to 80 percent of the compound could be recovered in the bound state. Specificity of the binding could not be adequately investigated in the time allotted in the grant study.

SECTION 3

EXPERIMENTAL PROCEDURES

RECOVERY OF ORGANICS FROM DILUTE AQUEOUS SOLUTIONS BY XAD-2 ADSORPTION

Background

Amberlite XAD resins (Rohm and Haas) are polystyrene divinylbenzene copolymers formed into beads (20-50 mesh) with a macroreticular structure (1-3). The beads allow a high flow rate and yet possess a large hydrophobic surface area (>300 m^2/g) (3). They are, therefore, capable of adsorbing large quantities of hydrophobic species from water.

There have been many reports describing the recovery of dissolved organics on XAD resins. Recovery of chlorinated biphenyls (4), chlorinated pesticides including dieldrin (5), surfactants (3), polycyclic aromatic hydrocarbons (1,2), phenols (5), and other classes of nonpolar organic compounds in yields greater than 80 percent from 50-500 ppb or lower in solutions in water or seawater have been described. The recovery of adsorbed compounds from the resin is simpler than that from activated carbon (2,5) and there is less chance for chemical conversion of the compounds prior

to analysis. A general method for recovery of organics from water using highly purified XAD-2 has been outlined in detail by Junk, et al. (5). Recently, XAD-2 has been used for recovery of mutagenic substances from the urine of cigaret smokers, indicating that even very impure fluids can be utilized (6).

XAD-2 Adsorption Method

The procedure which was used for recovery of organic carcinogens from seawater followed that of Junk, et al. (5). The XAD-2 resin obtained from Rohm and Haas was purified by sequential solvent extractions with methanol, acetonitrile, and diethylether in a Soxhlet extractor for 8 hours per solvent. The washed resin was stored in glass-stoppered bottles under absolute ethanol. The sorbent column was prepared by pouring a slurry of the resin in ethanol into the column (Bio-Rad glass Econo columns, 0.7 cm inside diameter x 10 cm were suitable for this purpose); the ethanol was subsequently replaced by water passed through a charcoal filter and glass distilled.

Sample solutions containing carcinogen were prepared using water from several sources: distilled water, Lake Pontchartrain water taken at New Orleans, Louisiana, and seawater collected from the Gulf of Mexico near Biloxi, Mississippi. The water was spiked with radiolabeled carcinogens at concentrations ranging from 3 to 800 parts per trillion (pg/liter). The radiolabeled carcinogens which were used were dieldrin-1,2,3,4,10-C-14 (specific activity, 1.34 mCi/mmole, California Bionuclear Corporation), dimethylnitrosamine [methyl-C-14] (specific activity, 3.7 mCi/mmole, Califor-

nia Bionuclear Corporation), benzo(a)pyrene-H-3 (ICN, specific activity, 24 Ci/mmole), and N-acetyl-2-aminofluorene [9-C-14] (specific activity, 26 mCi/mmole, Schwarz/ Mann). These carcinogens were chosen to provide examples of a chlorinated hydrocarbon pesticide (dieldrin), a nitrosamine (dimethylnitrosamine), a polycyclic aromatic hydrocarbon [benzo(a)pyrene] and an aromatic amine (N-acetyl-2-aminofluorene). All have been shown to be capable of binding to DNA (7). Dieldrin and benzo(a)pyrene are believed to be important contaminants of seawater (5,8).. Aromatic amines may contaminate seawater from effluents of dye manufacture, and nitroso compounds are ubiquitous environmental pollutants (8). Therefore, these classes of carcinogens should be illustrative of the problems which could arise in any potential scheme for isolating and separating organic carcinogens from seawater. These carcinogens may all require activation to exert carcinogenic effects. They have been shown to be mutagenic in <u>in vitro</u> tests and to be activated by microsomal oxidizing enzymes to species capable of binding to DNA. Furthermore, all have been shown to induce tumor formation in animals, although their carcinogenicity to humans is still unknown.

The dilute solutions were passed through the XAD-2 columns at flow rates between 3 and 18 ml/min. Solutions made in seawater and lake water were first passed through a glass fiber filter (Whatman GF/C) to remove suspended solids. The effluent water from XAD-2 adsorption was discarded. After the spiked water solution was passed, the column was washed with several column volumes of distilled water. The adsorbed carcinogen was then eluted with 4 to 5 column volumes of diethylether. The diethylether was evaporated in a stream of nitrogen, and the residue placed in a scintillation vial. The sample was counted in toluene scintillant (5 g PPO, 0.13

g POPOP per liter) in a Beckman LS 133 counter. Percent recovery was calculated from the amount of carcinogen recovered (cpm) and the amount initially dissolved. These values are given in Table 1. Varying the XAD-2 bed volume, flow rate, and carcinogen concentration had little effect on recoveries. Recovery was significantly less for dieldrin from lake water than from distilled water or seawater. Most of this loss was found to be due to adsorption of labeled dieldrin to particulates in the water which were removed by filtration. Such loss was not significant for the other carcinogens tested.

Due to the volatility of dimethylnitrosamine, the ether was not evaporated after the desorption of the dimethylnitrosamine from the XAD-2; instead, aliquots were assayed directly in the scintillation fluid. As can be seen in Table 1, recovery of the dimethylnitrosamine was less than 5 percent of the input amount. Therefore, binding of such species to XAD-2 could not be used to recover them efficiently from dilute solutions in water.

The results given in Table 1 confirm and extend those reported by other workers. Junk, et al. (5) have reported a 93 percent recovery on XAD-2 of dieldrin from a 20 ppt solution in water. Osterroht (2) showed that aldrin, an isomer of dieldrin, could be recovered in 58 percent yield from a 10 ppb solution in seawater. The polycyclic aromatic hydrocarbon phenanthrene was recovered from seawater in almost 100 percent yield from a 10 ppb solution but in lower yields from 100 ppb or 1 ppb solutions (62 percent and 57 percent, respectively). The above recoveries were measured using gas chromatography and comparison with standards instead of using radioactive samples as in the presently reported study. In the study by

TABLE 1. XAD ADSORPTION OF CARCINOGENS

Carcinogen	Carcinogen input (total cpm)	Water volume (liters)	Carcinogen concentration (ppt)	Water source	Solution flow rate (ml/min)	XAD resin volume (cm^3)	Carcinogen recovered (total cpm)	Recovery of carcinogen (percent)
dieldrin	3600	1	430	Distilled	13	5.03	2570	71.4
dieldrin	3600	1	430	Sea	8	8.05	2635	73.2
dieldrin	3600	3.78	114	Lake Pontchartrain	7	11	1660	46.1
benzo(a)pyrene	400000	1	5.2	Sea	7	16.6	296900	74
benzo(a)pyrene	80000	0.32	3.15	Lake Pontchartrain	3	13.3	48000	60
AAF*	51000	1	90	Lake Pontchartrain	3	14.1	40500	79.4
AAF	51000	1	90	Sea	3.4	15.1	51099	100
dimethyl-nitrosamine	82500	0.6	833	Distilled	6	12.1	4135	5
dimethyl-nitrosamine	82500	0.9	555	Sea	18	14	1500	2

*N-acetyl-2-aminofluorene

Yamasaki and Ames (6), recoveries were measured by the Salmonella/microsome (9) test. In the Yamasaki and Ames study, benzo(a)pyrene and N-acetyl-2-aminofluorene were recovered from urine using XAD-2 in yields of 19 percent and 89 percent, respectively, from 0.8 ppm solutions. The present results show that the N-acetyl-2-aminofluorene is recovered from seawater and lake water in high yields.

From the present studies and from the work of others it can be concluded that XAD-2 can be used to recover certain organic carcinogens from seawater in high yields. A possible exception is the recovery of more polar carcinogens such as dimethylnitrosamine. Yamasaki and Ames (6) found that certain metabolic derivatives of carcinogens (for example glucuronides) were not bound well by the nonpolar resin. The effect of environmental oxidation on polarity of polycyclic aromatic species and their ultimate ability to be recovered on XAD-2 has not been explored and would be an important factor in evaluating the usefulness of XAD-2 adsorption as the only method for concentrating organic carcinogens from dilute solution in seawater. Other possible methods for concentrating organics from marine waters, such as reverse osmosis, suffer from the fact that the maximum achievable concentration is 10- to 25-fold due to the increase in salt in the concentrate and the resulting increase in osmotic pressure. The use of bioadsorption by marine organisms such as the clam or mussel are potentially useful since 1000-fold concentrations of certain organics can be achieved (10). However, only small quantities of the concentrates can be obtained and these must be extracted from the tissue of the organism, thereby complicating the recovery process.

SEPARATION OF CARCINOGENS FROM NONCARCINOGENS BY DNA-BINDING

Current theoretical insight into the mechanism of carcinogenesis considers the first step in the process to result from the binding to and alteration of DNA by a carcinogen (7). It is now considered axiomatic that carcinogens are mutagens, compounds able to alter DNA thereby causing a permanent, hereditarily transmitted change in the genetics of the altered cell. Therefore, carcinogens must bind to DNA by either covalent or non-covalent attachment. If such binding is fairly specific for carcinogenic (and mutagenic) species, then at least a partial separation of such species from nonmutagenic species might be possible. Most carcinogens (often termed procarcinogens or promutagens) require metabolic activation before they can chemically alter DNA. The most common activation processes are oxidations by the microsomal mixed function monooxygenase system, e.g., arylhydrocarbon hydroxylase. Such functionalization is necessary before the carcinogen can bind covalently to DNA base nitrogens. The mechanism of oxidations have been widely discussed in the literature (11-13) and in certain cases presumed ultimate carcinogens identified from so-called proximate species. It is known that many proximate carcinogens can also bind noncovalently to DNA, although such binding may not result in mutagenesis (14).

In the present study, an investigation has been made of different conditions for interacting DNA with both activated and unactivated samples of the carcinogens benzo(a)pyrene, dieldrin, N-acetyl-2-aminofluorene, and dimethylnitrosamine. In each case, both total binding and total covalent

binding have been estimated. The question of specificity of the interaction (carcinogens versus noncarcinogens) has not been answered in this study.

DNA-Cellulose Adsorption of Carcinogens

DNA-cellulose chromatography has been used for the isolation and purification of DNA-binding enzymes and proteins. The use of immobilized DNA has at least two potential advantages over direct interaction of DNA with substrates in solution. First, a higher ratio of DNA to substrate is possible during the interaction process if the solution containing substrate is passed slowly onto a column of DNA-cellulose. This should maximize the amount able to bind. Second, the bound material (noncovalently bound only) can be selectively desorbed using certain elution solvents according to the binding strength for the individual component.

DNA-cellulose was prepared according to the procedure described by Alberts and Herrick (15). A solution of calf thymus or Salmon sperm DNA (Sigma), at 1-3 mg/ml in 0.01 M Tris-HCl, pH 7.4, 10^{-3}M EDTA (Tris-EDTA) was transferred to a beaker and Whatman CF-11 cellulose was added until the paste thickened. The mixture was allowed to sit at room temperature until dry. The dried mixture was ground to a powder and evaporated by lyophilization to remove any remaining water. The dry powder was suspended in Tris-EDTA for 24 hours at 4°C and washed twice to remove nonbound DNA. The DNA-cellulose was stored as a frozen slurry in Tris-EDTA. For the experiments described below DNA-cellulose was prepared with a DNA content of 0.09 to 0.48 mg DNA per packed ml of cellulose. The amount bound was determined by the uv adsorption (A_{260}) of an aliquot heated to 100°C for 20 min in Tris-EDTA to release the DNA from the cellulose.

The DNA-cellulose powder was loaded into Bio-Rad Econocolumns, 0.7 x 10 cm, in Tris-EDTA. As controls, columns were prepared with cellulose which had gone through the same preparative steps employed with the DNA-cellulose. The columns were washed thoroughly with Tris-EDTA buffer before adding solutions of the carcinogens.

Since the organic carcinogens chosen for the DNA-binding study all require activation by microsomal enzymes to exert their mutagenic effect, samples were added to DNA-cellulose with and without prior activation. The activation system used was that recommended by Ames et al. (9) for Salmonella/microsome testing. A liver homogenate was prepared from 200 g male Sprague-Dawley rats which were injected with 500 mg/kg arochlor 1254 five days prior to sacrifice. The livers were homogenized in 3 volumes of 0.15M KCl with a Tekmar Tissuemizer for 1 min at top speed. The homogenate was centrifuged at 900 x g for 10 min at 4°C in an International B60 centrifuge. The supernatant (S9) was collected and stored at -75°C in 1 ml aliquots. The activity of the S9 was checked by determining its ability to convert 5 µg benzo(a)pyrene to mutagenic products in the Ames Salmonella/microsome test. An aliquot of 0.05 ml S9 (0.5 ml S9 mix, see below) gave a 9-fold increase compared to control in revertant colonies for Salmonella strain TA98.

In the experiments in which the effect of S9 activation was tested, incubation (1 hour at 37°C) of the DNA-cellulose with carcinogen and S9 mix (contains per ml: 0.1 ml S9, 8 µmoles $MgCl_2$, 33 µmoles KCl, 5 µmoles glucose-6-phosphate, 4 µmoles NADP, and 100 µmoles sodium phosphate, pH 7.4) was performed in a batchwise manner in a screw cap culture tube instead of a column. Conditions were identical for samples not activated with microsomal mix, except that S9 was not included.

In the column experiments (no S9 activation), the column was further eluted with 5 column volumes of Tris-EDTA after the sample had passed into the column bed. This was followed by a linear gradient consisting of 5 column volumes of Tris-EDTA and DMSO. Finally, the column was rinsed with toluene until no further radioactivity appeared in the wash. The residual radioactivity on the column, if any, was determined by counting aliquots of the DNA-cellulose (or cellulose) and the solution obtained by heating the DNA-cellulose (or cellulose) at 100°C for 30 min in DMSO:Tris-EDTA (1:1). After these treatments, less than 1 percent of the input radioactivity was eluted from the column. A similar procedure was used in the batchwise DNA-cellulose studies except that washes after the buffer washes were with 100 percent DMSO. In these studies after each wash the cellulose was centrifuged down and the supernatant withdrawn. Washing with each solvent was continued until no further radioactivity was eluted.

The results of these studies are given in Table 2 for the column experiments and Table 3 for the batchwise incubation studies. In the column studies with dieldrin and benzopyrene (Table 2), most of the carcinogen (97 percent and 96 percent, respectively) remained bound to DNA-cellulose after the elution with buffer only, whereas for acetylaminofluorene much less (16 percent) was initially bound. The binding of dimethylnitrosamine was not tested. For acetylaminofluorene in the column studies (no S9 activation), following the DMSO and toluene washes, 96 percent of the input radioactivity was recovered. Approximately 70 percent of the benzopyrene remained bound to the DNA-cellulose after extensive washing with DMSO and toluene. As can be seen in Table 2, carcinogen association with cellulose not containing DNA also occurs. This material, however, is more readily

TABLE 2. DNA-CELLULOSE COLUMN CHROMATOGRAPHY OF ORGANIC CARCINOGENS

Carcinogen	Input quantity cpm (nmoles)	Flow rate (ml/min)	Cellulose volume (cm^3)	Total DNA present (mg)	Amount eluted with different solvents (cpm)			Total recovered (percent of input)	Amount left on column (percent of input)
					Tris-EDTA	DMSO gradient	Toluene		
dieldrin + S9	765000 (286)	0.06	5.0	1	20622	84000	11480	16	84
dieldrin	765000 (286)	0.06	5.0	1	18530	188352	24710	30	70
dieldrin (control)	765000 (286)	0.06	5.0	0	18410	392880	324390	96	4
benzo(a)pyrene	885800 (0.04)	0.03	5.0	0.45	34800	227192	14580	31	69
benzo(a)pyrene (control)	885800 (6.04)	0.03	5.0	0	19110	503102	21130	61	39
AAF*	2.6×10^6 (51)	0.03	4.3	1.6	2145000	400300	18500	96	4
AAF (control)	2.6×10^6 (51)	0.03	4.3	0	1589000	876000	62800	97	3

*N-acetyl-2-aminofluorene

TABLE 3. DNA-CELLULOSE BINDING OF ORGANIC CARCINOGENS (BATCHWISE INCUBATION STUDIES)

Carcinogen	Input cpm (nmoles)	Total DNA present (mg)	Amount eluted with different solvents (cpm)		Estimated percent of input sample bound to microsomes	Estimated percent of input bound to DNA-cellulose
			Tris-EDTA	Dimethyl sulfoxide		
dieldrin	320000 (120)	0.1	167172	148330	-	47
dieldrin + S9	320000 (120)	0.1	297612	16890	41	5
benzo(a)pyrene	840000 (0.04)	0.1	161547	674281	-	81
benzo(a)pyrene + S9	840000 (0.04)	0.1	608904	226924	53	27
N-acetyl-2-aminofluorene	1100000 (22)	0.1	882705	188118	-	18
N-acetyl-2-aminofluorene + S9	1100000 (22)	0.1	998633	72190	11	7

eluted by the organic solvent extractions. The results with dieldrin are similar to those with benzopyrene; most of the carcinogen is bound initially to either cellulose or DNA-cellulose. After elution with organic solvents most of the input radioactivity was recovered from the cellulose column but only 30 percent was recovered from the DNA-cellulose column. When S9 was included in the dieldrin sample, slightly more radioactivity was bound after the organic washes.

Somewhat different results were obtained when DNA-cellulose was allowed to interact with carcinogen in a batchwise manner. In these studies, following the DMSO extraction, almost all of the input radioactivity was not bound to the DNA-cellulose. Less than 1 percent of the radioactivity remained with the DNA-cellulose. The difference in the amount of material recovered in the Tris-EDTA washes for microsome-treated samples and samples not treated with microsomes is considered to represent the amount of material bound to the microsomes. This material would not be in contact with the DNA-cellulose (or cellulose) and therefore would be recovered in the supernatant. The amount bound to DNA-cellulose is considered to be the percent of the input radioactivity not eluted with Tris-EDTA, (i.e., the amount extracted by DMSO). This amount is not the amount bound to DNA, however, since in control studies most of this radioactivity was also retained by cellulose.

The results of DNA-cellulose binding clearly show that a column method is preferred to a batchwise method of interacting sample with the DNA since a tight binding is achievable by the former method but is not found for the latter. The chlorinated hydrocarbon, dieldrin, and the polycyclic aromatic, benzopyrene, are readily adsorbed onto DNA-cellulose and cellulose columns

from dilute aqueous solutions. They are not recovered from the DNA-cellulose following several organic solvent washes, indicating that they can bind tightly to the DNA in the DNA-cellulose. In the batchwise studies, almost 100 percent recovery of the bound compounds is possible with a DMSO wash indicating that they are less tightly adsorbed. N-acetyl-2-aminofluorene is not bound tightly in either method.

The potential use of DNA-cellulose binding as a method for separating carcinogens from noncarcinogens has not been assessed in these experiments so no clearcut conclusions in this regard can be drawn. However, since cellulose alone has an affinity for these carcinogens it is unlikely that specificity of interaction could be achieved by this method.

Adsorption of Carcinogens to Isolated Rat Liver Nuclei

Another method investigated for testing the binding of carcinogens to DNA was the interaction of arochlor 1254-induced rat liver nuclei with carcinogens. This method was chosen for two reasons: (1) Nuclei have been found to possess the metabolic activation enzymes necessary for conversion of inactive carcinogens to DNA-binding carcinogens (16). (2) The native state of the DNA in nuclei may allow the greatest possible interaction of sample with DNA. Since the DNA in nuclei is associated with chromatin proteins, such interaction may reflect the type of binding which would exist with DNA in the intact organism.

For this study the method of Bresnick et al. (16) was used. The incubation system included: sodium phosphate buffer, pH 7.4, 100 μmoles;

EDTA, 100 µmoles; glucose-6-phosphate, 18 µmoles; glucose-6-phosphate dehydrogenase, 6 units; NADPH, 1 µmole; $MgCl_2$, 3 µmole; rat liver nuclei, 5 mg nuclear protein; and radiolabeled carcinogen in a total volume of 2.0 ml. The mixture was incubated at 37°C for 30 min in tubes covered by aluminum foil to prevent light-induced reactions.

Nuclei were prepared from livers of rats injected intraperitoneally with arochlor 1254 5 days prior to sacrifice. The excised livers from the decapitated rats were washed in isotonic buffered saline, cut into small pieces with scissors and homogenized in a Potter-Elvehjem apparatus with a Teflon pestle using 20 up-and-down strokes and a medium speed setting. The homogenate was centrifuged 2 min at 2000 rpm at 5°C in an International centrifuge. The crude preparation of nuclei was further purified by layering the pellet suspended in 3 ml 1.2 M sucrose and 2 ml 0.01 M Tris·HCl, 0.01 M $MgCl_2$ pH 7.2 over a solution of 20 ml 1.2 M sucrose (17). The pellet was then taken up in 30 ml of 60 percent sucrose and centrifuged at 40000 x g for 60 min in an International B60 centrifuge. The nuclear pellet was resuspended in 1.0 M sucrose, 1 mM calcium acetate, rehomogenized, and centrifuged at 3000 x g for 5 min. The precipitate was used as the nuclei in the adsorption experiments described above.

After the incubation with carcinogen the nuclei were centrifuged at 3000 x g and the DNA recovered from the nuclear pellet by the following procedure: To the pellet was added 2 ml of 0.01 M Tris·HCl pH 8.0, 0.1 M EDTA, 0.25 M NaCl, 0.5 percent sodium dodecyl sulfate, 50 µg/ml proteinase K. The sample was incubated at 60°C for 16 hours. After cooling, the solution was extracted with shaking on a Burrell-Wrist Action Shaker with 2

ml of buffer-saturated phenol, 30 min. The aqueous (upper) phase was collected by centrifugation and the phenol layer washed with a small amount of buffer. The combined aqueous layers were reextracted with chloroform: isoamyl alcohol (24:1) until no protein interface was seen after centrifugation. The aqueous layer was then treated with 2 volumes of 0°C ethanol and the DNA spooled. The spooled DNA was redissolved in 0.01 M Tris·HCl pH 7.2; 10^{-4} M EDTA and aliquots were taken for scintillation counting.

In certain cases the DNA solutions were chromatographed in 0.01 M Tris·HCl pH 7.0, 0.1 percent SDS buffer on BioGel A5 columns to confirm that all of the radioactive label migrated with the void volume DNA peak. Furthermore, the combined column fractions containing the DNA peak were extracted with chloroform:isoamyl alcohol to verify that none of the radioactivity was associated with protein which would be extracted by such treatment. Results are given in Table 4. A representative profile of BioGel A5 chromatography is given in Figure 1 for benzo(a)pyrene-nuclei binding. These results show that as much as 18 percent of the input N-acetyl-2-aminofluorene can be recovered associated with DNA isolated from nuclei and that 54 percent of the input benzopyrene can be similarly recovered. Using similar methods, dieldrin and dimethylnitrosamine can be recovered in approximately 10 percent yield. The percent of the input radioactivity bound to the nuclei after the incubation period is given in Table 4. For benzopyrene and acetylaminofluorene almost 100 percent was initially bound, whereas for dieldrin and dimethylnitrosamine approximately 50 percent was initially bound.

TABLE 4. NUCLEI BINDING OF ORGANIC CARCINOGENS

Carcinogen	Input (cpm)	Input (ηmoles)	Amount associated with nuclei (cpm)	Percent of input associated with nuclei	Amount bound to DNA spooled from ethanol (cpm)	Percent of input associated with DNA
dieldrin	4×10^5	150	2.3×10^5	58	4×10^4	10
benzo(a)pyrene	6.9×10^6	0.32	6.7×10^6	97	1.9×10^6	27
N-acetyl-2-aminofluorene	1.6×10^6	31	1.5×10^6	94	3.3×10^5	18
dimethylnitrosamine	1.7×10^6	232	8.5×10^5	50	1.4×10^5	8

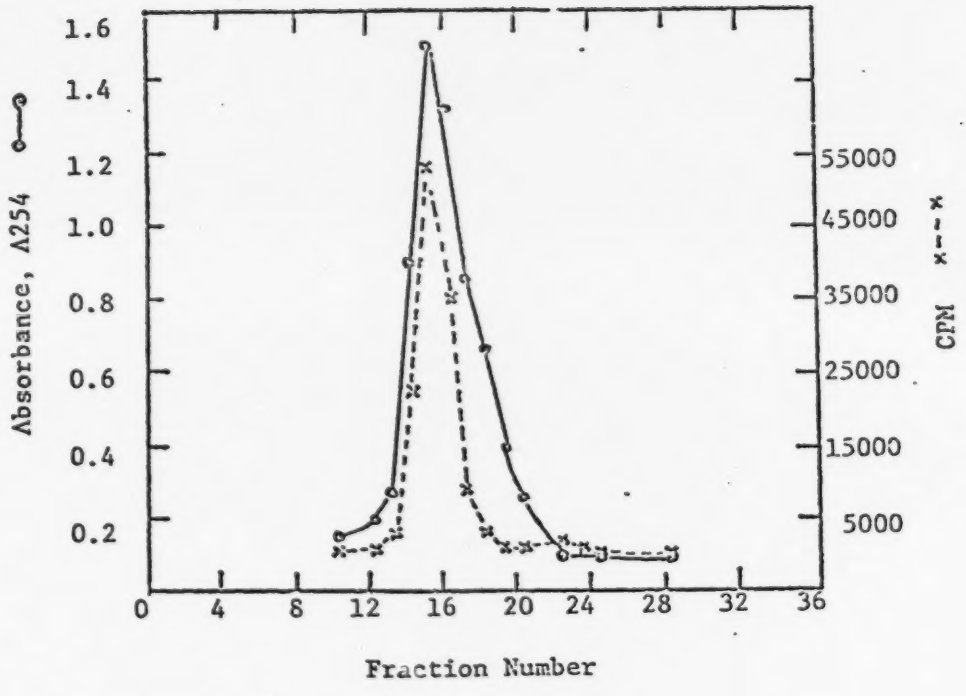

Figure 1. Binding of Benzo(a)pyrene to DNA - Nuclei Binding Method BioGel A5 Chromatography.

From these results it can be concluded that nuclei binding of the tested carcinogens occurs readily. Inclusion of S9 in the activation system was found to be unnecessary (results not shown). The present studies did not evaluate the specificity of nuclei binding with regard to noncarcinogens versus carcinogens so no conclusions can be drawn concerning this question. The advantage in using nuclei binding as a method for recovering biologically active substances from seawater concentrates is the relative ease of the method and its closer resemblance to the type of binding of such substances to DNA *in vivo*.

Direct Binding of Carcinogens to DNA

Previous studies by other workers have shown that polycyclic aromatic hydrocarbons (18) as well as chlorinated hydrocarbons (19) can bind to and alkylate DNA. In addition, it is well known that most carcinogens can noncovalently associate with DNA by intercalation or by binding to DNA phosphate (14). By such association DNA could potentially select for carcinogenic substances from those incapable (and presumably noncarcinogenic) of such interaction.

The simplest method for testing the binding of a substance to DNA is by direct interaction with DNA in the presence or absence of S9 activating enzymes. For the studies described below, the method of Gelboin (18) was used with only slight modification. For testing the binding in the presence of enzyme activation, rat liver S9 mix was used as described before. The sample was incubated with S9 mix (0.1 ml) at 37°C for 15 min in the presence of salmon sperm, highly polymerized, double-stranded DNA (0.125 mg). The

total volume of the incubation mixture was 1 ml. After incubation, the DNA was precipitated by addition of cold ethanol (2 volumes) and redissolved in 0.01 M Tris 10^{-3} M EDTA. This solution was extracted with chloroform:isoamyl alcohol (24:1) several times to remove protein and non-bound label. Following the extraction, the DNA was again isolated by ethanol precipitation. Aliquots of the resolublized DNA were determined for radioactivity.

The results of the direct binding studies are given in Table 5. All of the tested carcinogens are recovered with the DNA in good yields except dimethylnitrosamine. With dieldrin an increased yield (56 percent compared to 12 percent) was obtained when S9 was included but with benzo(a)pyrene a decreased yield was found (7.6 percent compared to 21 percent). This effect may be ascribed to activation of benzo(a)pyrene to metabolites incapable of DNA-binding. No effect of S9 was seen with N-acetyl-2-amino-fluorene. With dieldrin, because it is present in higher amounts than benzo(a)pyrene, some of the radioactivity may be associated with residual protein of the S9 mix not removed by the chloroform:isoamyl alcohol washes. Presumably, less of the dieldrin is activated to non-binding species than is the benzopyrene sample but dieldrin may be tightly associated with the microsomal protein. In general, the recoveries are similar to those found in the other DNA-binding methods (see comparison Table 7, p. 28).

Equilibrium Dialysis Binding of Carcinogens to DNA

As a final method tested for the recovery of certain organic carcinogens with DNA, the equilibrium dialysis of carcinogen solutions versus DNA in a dialysis tube was measured. This method was studied for comparison only

TABLE 5. DIRECT BINDING OF DNA WITH CARCINOGENS

Carcinogen	S9	Input (cpm)	Input (nmoles)	Amount bound (cpm)	Percent of input amount bound
dieldrin	+	19160	7.5	11173	56
	-	22900	8.6	2700	12
benzo(a)pyrene	+	85500	0.004	6534	7.6
	-	244700	0.012	51839	21
N-acetyl-2-aminofluorene	+	397950	7.4	19364	4.9
	-	519500	9.7	24416	4.7
dimethylnitrosamine	+	418650	54	126	0.03
	-	438000	60	85	0.02

and has little practical relevance due to the long time (days) necessary to achieve equilibrium.

In this procedure, 0.25 to 0.50 μCi of radiolabeled carcinogen is dissolved in 120 ml of distilled water and added to a cylinder. A solution of calf thymus, highly polymerized, double-stranded DNA (6 ml containing 11 mg DNA) was placed in a dialysis sack (12000 molecular weight cutoff) closed with Spectrum clips.

The dialysis sack is suspended in the cylinder and the fluid stirred. Aliquots were taken at intervals until equilibrium was achieved. Controls with no DNA in the dialysis sack were run to ascertain losses of sample to the walls and to measure the normal dialysis equilibrium. Any preferential migration of the carcinogen into the dialysis bag over the control is a measure of carcinogen binding by the DNA. Correction for the loss of net dialysis volume due to the DNA solute has not been made. To confirm that the binding was strong, dialysis of the equilibrated solution against buffer was performed until no further radiolabel was lost from the dialysis bag. The remaining amount in solution is that bound to DNA.

The results of the equilibrium dialysis studies are given in Table 6. In most studies equilibrium was achieved after 4 to 6 days of dialysis. Equilibrium dialysis studies were performed without prior activation of carcinogens by S9 microsomes. From a measurement of material balance, losses of carcinogen to the walls and dialysis tubing were approximately 10-20 percent of the total input radioactivity. Dimethylnitrosamine was not tested in these studies.

In Table 6, the amount bound to DNA after equilibration of the carcinogen solution outside the dialysis bag with the DNA solution inside the dialysis bag is given as the cpm/ml (inside) minus the cpm/ml (outside) times the volume of the DNA solution. The cpm remaining inside the bag after exhaustive dialysis against buffer (0.01 M Tris pH 7.0, 10^{-3} M EDTA) is also given in Table 6. Using the known specific activity of the carcinogen, the amount bound per mg DNA can be calculated. This calculation uses the amount associated with the DNA after exhaustive dialysis to remove extraneous counts. The controls were run under identical conditions except that buffer was added to the dialysis sack instead of DNA solution. The controls at equilibrium gave identical amounts of radioactivity inside and outside the bag. In addition, all radioactivity inside was lost upon dialysis versus buffer.

The results show that most of the benzopyrene which is bound to the DNA at equilibrium remains bound after dialysis, whereas for dieldrin and N-acetyl-2-aminofluorene only one third or one fourth, respectively, remains bound. The percent of the input radioactivity bound to DNA can be calculated. In the equilibrium binding studies 1 percent of the dieldrin, 35 percent of the benzo(a)pyrene and 1 percent of the N-acetyl-2-aminofluorene remains bound to the DNA. Except for benzopyrene, such binding is less than that for the other DNA interaction systems.

Comparison of the DNA-binding Methods

In Table 7 an attempt has been made to compare the different methods used to recover carcinogen associated with DNA. The percent of the input

TABLE 6. EQUILIBRIUM DIALYSIS BINDING OF DNA WITH CARCINOGENS

Carcinogen	Input (cpm)	Input (ηmoles)	Amount of DNA (mg)	Amount bound to DNA at equilibrium (cpm)	Amount remaining bound after dialysis against buffer (cpm)	Amount bound per mg DNA (ρmoles)
dieldrin	198960	75	16	4770	1786	43
benzo(a)pyrene	214030	0.011	11	110184	74030	4
N-acetyl-2-amino-fluorene	752160	14	16	40188	10410	17

TABLE 7. COMPARISON[a] OF DNA BINDING METHODS

Method	Amount Bound to DNA							
	Dieldrin		Benzo(a)pyrene		AAF*		Dimethylnitrosamine	
	percent of input (cpm)	µmole mole DNA phosphate	percent of input (cpm)	µmole mole DNA phosphate	percent of input (cpm)	µmole mole DNA phosphate	percent of input (cpm)	µmole mole DNA phosphate
DNA-cellulose (column)	66	63000	30	9	1	110	NT[b]	NT
DNA-cellulose[c] (batchwise)	47	187000	81	100	18	13000	NT	NT
nuclei binding	10	19000	27	153	18	8000	8	24000
direct binding	12	2700	21	7.6	5	1100	0.02	31
equilibrium binding	1	14	35	1.3	1	5.7	NT	NT

*N-acetyl-2-aminofluorene

[a]Comparison is made only of samples not treated with S9.

[b]NT = not tested

[c]Amount bound to cellulose alone was not subtracted.

amount bound and the amount of carcinogen bound per mole DNA phosphate in the different binding studies have been compared. In the DNA-cellulose, batchwise binding studies the amount associated with cellulose has not been distinguished in Table 7 from the amount possibly bound to DNA. As a means of carcinogen recovery, nuclei binding and DNA-cellulose binding are to be preferred to direct or equilibrium DNA binding. However, the binding of carcinogen to cellulose or protein in these methods would render them far less specific for the separation of carcinogens from other related organic species which would presumably bind equally well. In the DNA-cellulose studies, specific recovery of the DNA was not performed in the same way (chloroform extraction, agarose chromatography) as for the other methods. However, the binding to cellulose and DNA-cellulose is sufficiently great to prevent recovery by toluene or DMSO extraction.

In the nuclei binding and direct binding studies, carcinogen associated with the DNA was recovered by methods used to purify DNA from proteins. Much greater binding was found in the nuclei studies compared to direct-binding studies, particularly for dimethylnitrosamine. This difference could be partly due to inadequate purification of the nuclei-derived DNA. The presence of DNA-associated proteins could cause an increase in the amount of carcinogen recovered in these studies. From the S9 microsome activation studies the conclusion can be made that these carcinogens can bind readily to protein.

The studies which have been described used carcinogens at the nmolar concentrations associated with the radioactive specific activity as received. Therefore, the maximum possible binding has not been explored. The effect

of concentration on DNA-binding would certainly be valuable for future discussion of this method as a means of recovery of organic carcinogens from solutions. Since the different carcinogens, particularly benzo(a) pyrene, are at different concentrations a comparison of the binding strength of the different types of carcinogens is not possible. Benzo(a)pyrene was at such high specific activity that its maximal binding probably was not achieved. The fact that dieldrin shows the greatest binding may be the consequence of its much lower specific activity rather than an intrinsically higher association constant.

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